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(54) Title: METHODS OF <i>IN SITU</i> MODIFICATION OF PLANT GENES		
(57) Abstract <p>A method of producing plants which exhibit an agronomically desirable trait comprises mutating or otherwise modifying <i>in situ</i> in a plant cell at least one gene which when modified is responsible for providing the said trait and regenerating from a cell exhibiting the said trait fertile morphologically normal whole plants, and is characterised in that a polynucleotide is introduced into the plant cell, the said polynucleotide comprising at least one region which is substantially complementary to at least one region in the gene, which gene region when mutated or otherwise modified provides for the agronomically desirable trait, the region in the said polynucleotide containing at least one base mismatch in comparison with the like region in the said gene, so that the region in the said gene is altered by the DNA repair/replication system of the cell to include the said mismatch.</p>		

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METHODS OF IN SITU MODIFICATION OF PLANT GENES

The present invention relates to the production of plants which exhibit certain desirable agronomic traits and which are produced by a non-biological process not obligatorily involving transformation or transgenesis (although these techniques can be used).

According to the present invention there is provided a method of producing plants which exhibit an agronomically desirable trait comprising mutating or otherwise modifying *in situ* in a plant cell at least one gene which when modified is responsible for providing the said trait and regenerating from a cell exhibiting the said trait fertile morphologically normal whole plants, characterised in that a polynucleotide is introduced into the plant cell, the said polynucleotide comprising at least one region which is substantially complementary to at least one region in the gene, which gene region when mutated or otherwise modified provides for the agronomically desirable trait, the region in the said polynucleotide containing at least one base mismatch in comparison with the like region in the said gene, so that the region in the said gene is altered by the DNA repair/replication system of the cell to include the said mismatch.

By "gene" is meant a polynucleotide comprising - contiguously - a sequence to which an RNA polymerase is capable of binding (promoter), an RNA encoding sequence and a transcription termination sequence. At least one of the following regions of the gene may be mutated or otherwise modified: promoter, RNA encoding sequence or transcription terminator. In a preferred embodiment of the method a transcription enhancing region associated with the gene is mutated or otherwise modified *in situ*.

Whilst the said trait could be an improved resistance to insects and/or fungal or bacterial infections, it is particularly preferred that the trait is herbicide resistance. The herbicides to which plants resulting from the method according to the invention are rendered resistant, or to which the said plants are tolerant or exhibit relatively improved resistance, are selected from the group consisting of paraquat; glyphosate; glufosinate; photosystem II

synthase; herbicides which inhibit acetyl CoA carboxylase; herbicides which inhibit protoporphyrinogen oxidase; herbicides which inhibit phytoene desaturase; herbicides which

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inhibit hydroxyphenylpyruvate dioxygenase and herbicides which inhibit the biosynthesis of cellulose.

Plants which are substantially "tolerant" to a herbicide when they are subjected to it provide a dose/response curve which is shifted to the right when compared with that provided by similarly subjected non tolerant like plants. Such dose/response curves have "dose" plotted on the x-axis and "percentage kill", "herbicidal effect" etc. plotted on the y-axis. Tolerant plants will require more herbicide than non tolerant like plants in order to produce a given herbicidal effect. Plants which are substantially "resistant" to the herbicide exhibit few, if any, necrotic, lytic, chlorotic or other lesions when subjected to the herbicide at concentrations and rates which are typically employed by the agrochemical community to kill weeds in the field. Plants which are resistant to a herbicide are also tolerant of the herbicide. The terms "resistant" and "tolerant" are to be construed as "tolerant and/or resistant" within the context of the present application.

The skilled man will appreciate that the plant material in which the *in situ* modification is performed may have been prior transformed with a gene providing for resistance to insects, fungi, and/or herbicides, or with a gene capable of providing plants regenerated from such material with, for example, an increased capacity to withstand adverse environmental conditions (improved drought and/or salt tolerance, for example) in comparison with plants regenerated from non-transformed like material.

At least one region of the polynucleotide may consist of RNA. The polynucleotide other than that comprised by the said at least one region may consist of DNA. The polynucleotide may consist of between about 30 and 250 nucleotides. In a more preferred embodiment of the polynucleotide it consists of between 50 and 200 nucleotides.

The protein encoding region of the gene may encode an enzyme selected from the group consisting of EPSPS, GOX, PAT, HPPD, ACC, ALS, BNX and protox and known mutated or variant forms thereof. In particular, the said gene may encode an EPSPS enzyme as depicted, for example, in SEQ ID Nos. 1 or 10. It is preferred that the EPSPS enzyme has least the residues Thr, Pro, Gly and Ala at positions corresponding to 174, 178, 173 and 264 with respect to the EPSPS depicted in SEQ ID No. 2, and that the said mismatch results in at least one of the following modifications in the EPSPS enzyme in comparison with the native sequence:

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- (i) Thr 174 - Ile
- (ii) Pro 178 - Ser
- (iii) Gly 173 - Ala
- (iv) Ala 264 - Thr

5 wherein (i) Thr 174 occurs within a sequence comprising contiguously Ala -Gly-Thr-Ala-Met; (ii) Pro 178 occurs within a sequence comprising contiguously Met-Arg-Pro-Leu-Thr; (iii) Gly 173 occurs within a sequence comprising contiguously Asn-Ala-Gly-Thr-Ala; and (iv) Ala 264 occurs within a sequence comprising contiguously Pro-Leu-Ala-Leu-Gly.

Alternatively, and/or additionally, the mismatch may result in replacement of the
10 terminal Gly residue within the sequence motif Glu-Arg-Pro-AA1-AA2-AA3-Leu-Val-AA4-AA5-Leu-AA6-AA7-AA8-Gly- in a region of the EPSPS enzyme corresponding to that spanning positions 202 to 216 in SEQ ID No. 2 by either an Asp or Asn residue.

The plant cell to which the method of the invention is applied may be a cell of a plant selected from the group consisting of canola, sunflower, tobacco, sugar beet, cotton, maize,
15 wheat, barley, rice, sorghum, tomato, mango, peach, apple, pear, strawberry, banana, melon, potato, carrot, lettuce, cabbage, onion, soya spp, sugar cane, pea, field beans, poplar, grape, citrus, alfalfa, rye, oats, turf and forage grasses, flax and oilseed rape, and nut producing plants insofar as they are not already specifically mentioned

The plant cell may be converted into a protoplast prior to the *in situ* mutation or
20 modification of the gene - or transcriptional enhancing regions associated therewith - which when modified provides for the agronomically desirable trait.

The invention further includes plants which result from the method disclosed herein, as well as the progeny and seeds of such plants, and plant material derived from such plants, progeny and seeds.

25 The invention still further includes a method of selectively controlling weeds in a field, the field comprising plants as disclosed in the preceding paragraph and weeds, the method comprising application to the field of a herbicide to which the said plants have been rendered resistant. Insecticidally effective amounts of insecticides and/or fungicidally

The invention will be further apparent from the following description taken in conjunction with the associated sequence listing.

SEQ ID No. 1 shows the cDNA from *Petunia* encoding an EPSPS enzyme. Nucleotides 28 to 243 encode the transit peptide responsible for targeting the EPSPS enzyme encoded by nucleotides 244 to 1578 to the chloroplast. SEQ ID No. 2 shows the translational product of the sequence depicted in SEQ ID No. 1. Protein having the sequence of amino acid residues 1 to 72 constitutes the chloroplast transit peptide; protein having the sequence of amino acids 73 to 516 constitutes the EPSPS enzyme. SEQ ID Nos 3 and 4 depict peptides encoded by sequences (SEQ ID Nos 5 and 7) within exons 2 and 4 respectively of the *Brassica napus* EPSPS gene. Sequence ID Nos. 6 and 8 are mixed ribo-deoxyribonucleic acid sequences which are capable of forming duplexes with the sequences depicted in SEQ ID Nos. 5 and 7 respectively. SEQ ID Nos. 28 and 29 are sequences which are comprised by the sequences depicted in SEQ ID Nos. 5 and 7 respectively. SEQ ID Nos. 9 and 10 depict respectively (i) the genomic DNA from *Brassica napus* which encodes a spliced RNA encoding an EPSPS enzyme, and (ii) the amino acid sequence of the said *Brassica* EPSPS enzyme. SEQ ID Nos 11 - 27 depict mixed oligonucleotides (ie containing both ribo and deoxyribonucleotides) comprising sequences (marked with asterixes in the reiteration of the sequences in the corresponding Examples) capable of causing mutations in the gene to which the oligonucleotide is targeted. The oligonucleotides depicted in SEQ ID Nos 11 to 27 are all designed to cause plant material into which they are incorporated to become resistant to herbicides, such as glyphosate and chlorsulfuron, by causing the gene encoding the proteinaceous target for the herbicide to become mutated so that the target is no longer sensitive to the herbicide. Should there be a discrepancy between the sequences depicted in the sequence listings and those corresponding sequences depicted in the Examples, the Example sequences are definitive. In the Examples sequences depicted in lower case are RNA and those in upper case are DNA.

25 Methods

Polynucleotides Mixed ribo-deoxyribonucleic acids are synthesised by synthetic and semisynthetic methods known to those skilled in the art (for example Scaringe, S.A. et al (1990), *Nucleic Acids Research* 18:5433-5441; Usman, N. et al (1992) *Nucleic Acids Research* 20:665-6699 and Swiderski, P.M. et al (1994) *Anal. Biochem.* 216:83-88. Eric B. Kmiec (1996) United States Patent 5,565,350). Mixed ribo-deoxyribonucleic acids are synthesised using natural nucleotides, or, in some cases, preferably with 2'-O methylated ribonucleotides. Additionally or alternatively the phosphodiester bonds of the nucleic acid

are replaced by phosphorothiodiesters or methylphosphonodiester. Additionally or alternatively arabinose-containing nucleotides are also used.

A duplex nucleic acid in which deoxyribonucleotides and ribonucleotides correspond with each other is termed a hybrid-duplex. When two strands form a region of duplex nucleic acid for less than all of their bases the resultant molecule is termed a heteroduplex. Two strands of a duplex can be linked by an oligonucleotide linker region to form a single polymer. The bases in the linker region are not Watson-Crick paired. A heteroduplex in which the first and second strands are portions of a single polymer is termed a hairpin duplex.

The mixed ribo-deoxyribonucleic acid useful in the present invention has at most one 3' end and one 5' end. It is constructed to contain at least one region of at least one or more - usually three to four - bases that are not Watson-Crick paired. These unpaired regions form linker regions between two strands of Watson-Crick paired bases. It is preferred that the bases of the linker regions are deoxyribonucleotides.

In a preferred embodiment, the mixed ribo-deoxyribonucleic acid is constructed having two linkers arranged a) such that substantially all of the remaining bases are Watson-Crick paired and b) such that the 3' and 5' ends of the polymer are Watson-Crick paired to adjacent nucleotides of the complementary strand. These can be ligated to form a single continuous circular mixed ribo-deoxyribonucleic acid polymer.

In the present invention, the mixed ribo-deoxyribonucleic acid is used for the purpose of specifically introducing alterations (a mutation) into a target gene. The genetic site of alteration is determined by selecting a portion of the mixed ribo-deoxyribonucleic acid to have the same sequence as (to be homologous with) the sequence of the target site, hereinafter termed a homologous region. The area of differences between the sequence of the mixed ribo-deoxyribonucleic acid and the target gene is termed the heterologous region. Preferably there are two homologous regions in each mixed ribo-deoxyribonucleic acid flanking an interposed heterologous region, all three regions being present in a single continuous duplex nucleic acid. Furthermore each homologous region contains a portion of

A pair of homo-duplex may be placed within a region of hybrid duplex to allow ligation of the

3' and 5' ends to each other. The total length of the two homologous regions is at least 20 base pairs and preferably is between 40 and 60 base pairs.

A region of homo-duplex can be disposed between the hybrid-duplex/ homologous regions of the vector. The interposed homo-duplex can contain the heterologous region.

- 5 When the heterologous region is less than about 50 base pairs and preferably less than about 20 base pairs, the presence of an interposed homo-duplex is optional. When the heterologous region exceeds about 20 base pairs, an interposed homo-duplex is preferred.

The change to be introduced into the target gene is encoded by the heterologous region. The change to be introduced may be a change in one or more bases of the target gene
10 sequence or the addition of one or more bases.

Design of polynucleotides to achieve in situ mutagenesis of EPSP synthase in *Brassica napus* variety Westar.

- It is known that the combination of mutations G101A and A192T in a *Petunia* EPSPS can provide for resistance to glyphosate, whilst maintaining a low K_m for PEP. The equivalent residues in the sequence of the *B. napus* enzyme are (1) the
15 second glycine occurring within the sequence LGNAGTAMRPLT (SEQ ID No. 3) where this G is amino acid 173 wherein amino acid 1 is the starting methionine of the transit peptide and (2) the third alanine occurring within the sequence MAAPLALGDVEI (SEQ ID No. 4) and consequential having the residue number 264.

- The glycine residue occurs within exon 2 (part of which is shown below and is
20 depicted as SEQ ID No. 5), the DNA coding sequence in the region being:

L G N A G T A M R P L T

ATTGAGTTGTACCTTGGGAATGCAGGAACAGCCATGCGTCCACTCACCGCTGCA

An example of the desired mutation is GGA ---> GCA

- The mixed ribo-deoxyribonucleic acid designed to elicit this change includes, for
25 example, on one of its strands, a sequence comprising mainly of RNA which is complementary to all or part of the above DNA sequence. This RNA is interposed by a short region of DNA also complementary with the corresponding region of the above DNA sequence except for the inclusion of the specific mismatch of having a guanosine base opposite the guanosine base within the target GGA codon. A suitable mixed ribo-
30 deoxyribonucleic acid could thus include all or part of the following sequence (depicted as SEQ ID No. 6 in the sequence listing). Note that RNA sequence is marked in bold.

- 7 -

TTGTACCTTGGGAATGCAGGAACAGCCATGCCGTCCACTC
AACAUGGAACCCUUACGTCGTTGUCGGUACGCAGGUGAG

The corresponding alanine residue occurs within exon 4 (part of which is shown below and is depicted as SEQ ID No. 7).

5 M A A P L A L G D V E I
 ACTGCCCTCCTCATGGCAGCTCCTTTAGCTCTTGGAGACGTGGAGATTGAGATCATT

An example of the desired mutation is GCT ---> ACT. The mixed ribo-
 deoxyribonucleic acid designed to elicit this change includes, for example, on one of its
 strands, a sequence comprising mainly of RNA which is complementary to all or part of the
 above DNA sequence. This RNA is interposed by a short region of DNA also
 10 complementary with the corresponding region of the above DNA sequence except for the
 inclusion of the specific mismatch of having a thymine base opposite the guanosine base
 within the target GCT codon. The desired polynucleotide thus includes all or part of the
 RNA sequence depicted below and in SEQ ID No. 8. Note that RNA sequence is marked in
 15 bold.

TCCTCATGGCAGCTCCTTTAGCTCTTGGAGACGTGGAGATT
AGGAGUACCGUCGAGGAAATTGAGAACCUCUGCACCUCUAA

Besides the examples detailed above there will of course be many other specific
 changes which could be introduced into those sequences which regulate gene expression and
 20 for which polynucleotides can easily be designed by methods directly analogous to that
 described above and which, for example, could be useful to achieve increased expression of
 EPSPS. The skilled man will appreciate that many methods could be used to specify those
 changes potentially useful for increasing the expression of EPSPS. For example:

(1) The skilled man will be aware of instances of resistance to glyphosate having
 25 occurred in both field populations of weeds (e.g. Australian *loium*) and upon continuous
 selection of cultured plant cells (e.g. Hollander-Czytko et al (1988) in *Plant Mol. Biol.* 11,
 215-220; Hollander-Czytko et al (1992) *Plant. Mol. Biol.* 20, 1029-1036) or, for example,
 cultivars of birdsfoot trefoil (Boerboom et al (1990) *Weed. Sci.*, 38, 463-467) upon

Corydalis sempervirens (Hollander-Czytko et al (1988) in *Plant Mol. Biol.* 11, 215-220) a
 30-40 fold increase in the cellular content of EPSP synthase and an 8-12 fold increase in
 transcript levels was observed. That was a 100 fold increase in the EPSP synthase gene

It is a routine matter in all of the above examples using methods known to the skilled man to isolate cDNA encoding the EPSP synthases, to use these cDNA's as probes to identify clones from genomic libraries and to sequence the corresponding EPSP synthase genes and their 5' upstream and 3' downstream regions. Alternatively, genomic sequences
5 may be isolated directly using heterologous probes and/or combinations of degenerate and inverse PCR. By comparing the sequences so obtained from 'high EPSP synthase expression' lines of plants, cultivars or plant cells with the appropriate unselected controls the specific mutation(s) responsible for conferring high expression of EPSP synthase will be identified.

10 (2) Another example of a suitable method for identifying mutations potentially useful for increasing the expression of EPSP synthase is to directly select various lines of cultured plant cells or protoplasts from plant species of interest (e.g. *Brassica napus*) on increasing concentrations of glyphosate. This can be done with or without the addition of a suitable chemical mutagen. Glyphosate-tolerant lines so obtained are analysed for
15 expression of EPSP synthase, for the level of translatable EPSP synthase gene transcript (e.g. by Northern analysis) and for possible amplification of the EPSPS gene (e.g. by Southern and dot blot analysis). Cell lines of particular interest would be those where EPSP synthase was overexpressed and where this increase could not be accounted for through gene amplification. Identification of the specific mutation(s) responsible for conferring high
20 expression of EPSP synthase are then identified as described in (1) above.

(3) A further example of a method useful to specify mutations causing high expression of EPSPS comprises (a) subcloning the plant EPSP synthase promoter, 5' upstream sequence region, translational start region and sequence encoding the N-terminus region of EPSP synthase into a translational fusion construct directing the synthesis of a
25 suitable and easily measurable reporter gene such as (Beta glucuronidase) (b) further cloning this into a shuttle vector containing an origin for replication in *E. coli* and also designed for site specific integration into the yeast genome (YIP), or the genome of any other suitable test cell, such that integration into a specific location can be positively selected, by for example, complementation of an auxotrophic mutation. A library of many variants specifically within
30 the promoter and 5' upstream region of the so-designed shuttle vector is then created by mutagenesis through, for example, Mn²⁺-poisoned PCR of the region and maintained in *E. coli*. Members of the library are then tested by transformation into yeast. The best

expressers in yeast are identified by increased expression of the reporter gene. The integrated DNA from these high expresser lines is then extracted, sequenced and compared with the original sequence in order to identify those specific mutation(s) which conferred increased expression. Such mutations may affect conserved domains within the promoter which bind the transcriptional activators required for gene expression. Studies of this sort may teach those skilled in the art to modify the equivalent conserved regions in other crop plant species, thus enabling the technology to be applied broadly.

The polynucleotides comprising the RNA sequences disclosed above are transfected into protoplasts of *Brassica napus* which are then cultured and subjected to the herbicide glyphosate at concentrations which are sufficient to kill like protoplasts which have not been transfected and like protoplasts which have been transfected but with a polynucleotide not comprising regions designed to elicit a mutation in the *Brassica* genome. Those transfected protoplasts which survive the herbicide at concentrations which kill the control protoplasts are regenerated into plants using known means. The increased resistance to the herbicide of the thus regenerated plants is inherited in a Mendelian manner amongst the progeny of these plants.

The skilled man will appreciate that the invention is not limited to that specifically described above in respect of the production of glyphosate resistant *Brassica napus*. For plant species for which the EPSP synthase gene sequence(s) are already available on public databases the RNA and DNA elements of the polynucleotides can easily be designed by a method directly analogous to that described for *B. napus*. Polynucleotides comprising these RNA and DNA elements can then be introduced into regeneratable plant material from other species. Moreover, the skilled man is capable of designing:

(i) polynucleotides for the *in situ* mutagenesis of the DNA bases flanking the translational start site to improve post transcriptional efficiency of expression of EPSP synthase in plants, for example *Brassica napus* variety *Westar*. The consensus sequences for the regions immediately surrounding the translational start sites in animals (M Kozak, 1986, Cell, 44, 283-292) and plants (G Heidecker and J Messing, 1986, Ann. Rev. Plant Physiol

be improved *in situ* by designing mixed ribo-deoxyribonucleic oligonucleotides to make the

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desired mutational changes, at positions -3 and + 6 as shown below. Note that conserved consensus sequences are underlined.

	-4	-3	-2	-1	+1	+2	+3	+4	+5	+6
B. napus	<u>A</u>	T	C	A	<u>A</u>	<u>T</u>	<u>G</u>	<u>G</u>	<u>C</u>	G
Consensus	<u>A</u>	<u>A</u>	C	A	<u>A</u>	<u>T</u>	<u>G</u>	<u>G</u>	<u>C</u>	<u>T</u>

It will be obvious to those skilled in the art that this approach need not be confined to the EPSP synthase gene from *B. napus*, but may be applied to any plant species in which an increase in expression of the target gene is sought.

ii) polynucleotides for the *in situ* mutagenesis of the DNA bases to achieve an increase in transcriptional efficiency of expression of EPSP synthase. An approach similar to that described above may be adopted to achieve an enhancement in the rate of transcription of EPSP synthase genes by mutating bases at the "TATA" box region upstream from the transcription start point, and at the transcription start point itself. Identification of the transcription start point is identified using techniques, such as primer extension analysis, known to those skilled in the art. The "TATA" box is generally found 16-54 bases upstream of the transcriptional start. Consensus sequences have been published for plant transcription start point (V Pautot et al., 1989, *Gene*, 77, 133-140)

Plant Consensus CTCATCA

and "TATA" box regions (V Pautot et al., 1989, *Gene*, 77, 133-140)

Plant Consensus TCACTATATATAG

In both cases highly conserved bases are underlined. Comparisons between the consensus and native sequences of target EPSP synthase genes will enable bases suitable for mutational change to be identified.

(iii) polynucleotides for *in situ* mutagenesis to alter expression of EPSP synthase in plants, for example *Brassica napus* variety *Westar*.

Such designed polynucleotides can be introduced into totipotent plant material by known means which is then regenerated into plants which are subjected to a selection procedure to isolate those that exhibit the desired trait.

The skilled man will appreciate that directly analogous methods to those described above for EPSP synthase and glyphosate could be applied to other combinations of selecting herbicide and target gene where the aim is to specify mutations conferring over-expression.

The invention will be further apparent from the following Examples. Throughout the Examples the expression "selecting concentrations" of herbicide is present. By this is meant a concentration of herbicide which is sufficient to kill non-transformed material, or material which otherwise does not contain the oligonucleotides which are contained within like experimental material. The skilled man will know what those concentrations are having regard to the specific circumstances relating to his particular germplasm, transformation protocols and the expected variation between replicate procedures. The oligonucleotides shown below (SEQ ID Nos 11 to 27) are all synthesised according to Yoon *et al.* (1996). In each of the Examples where the constructs contain bases depicted in lower case, the sequence comprising such bases is to be understood as being RNA, and sequences comprising bases depicted in upper case as being DNA.

Example 1 This Example demonstrates the production of corn (maize) which is resistant to the herbicide chlorsulfuron.

[illegible]

The above oligonucleotide (SEQ ID No. 11) conveniently may be introduced into corn using silicon carbide whiskers, pollen harbouring the oligonucleotide or *via* pollen tubes.

Whiskers The so called whiskers technique is performed essentially as described by Frame *et al.*, (1994 Plant J. 6 941 -948). The oligonucleotide (1-100 µg) depicted in SEQ ID No.11 is added to the whiskers and used to transform A188 x b73 cell suspensions. The oligonucleotide(s) may be co-incubated with plasmids comprising sequences encoding proteins capable of forming recombinase complexes in plant cells such that recombination is catalysed between the oligonucleotide and the target sequence. Plant regeneration is performed using selective concentrations of chlorsulfuron in place of bialaphos. Plants are

emergence.

Pollen transformation Maize pollen is bombarded with gold particles by techniques known to the skilled man. Gold particles are coated with the oligonucleotide depicted in SEQ ID No. 11. The oligonucleotides may be co-incubated with plasmids comprising sequences encoding proteins capable of forming recombinase complexes in plant cells such that recombination is catalysed between the oligonucleotide and the target sequence. Suitable bombardment methods vary in precise detail but the basic procedure is well known to the skilled man and it is thus not necessary to describe it here. Bombarded pollen is applied to receptive silks of detassled plants. Sufficient replicas are performed to pollinate a large number of plants (typically up to 500). Progeny of the plants are screened for chlorsulfuron resistant members of the population by spraying with selecting concentrations of chlorsulfuron.

Pollen tube mediated transformation Emasculated corn plants are used. Wild type pollen is applied to pollination receptive silks. After between 30 min to 6 hours the silks are cut to within one cm of the base. The above SEQ ID No. 11 oligonucleotide (1-100 µg/ 10 µl in TE) is applied to the cut surface using a 1 ml syringe and needle such that the surface is completely covered. The oligonucleotides may be co-incubated with plasmids comprising sequences encoding proteins capable of forming recombinase complexes in plant cells such that recombination is catalysed between the oligonucleotide and the target sequence. The plants are then grown in a green house with an initial humidity of about 75 %. Progeny of the plants are screened for chlorsulfuron resistant members of the population by spraying with selecting concentrations of the herbicide.

Plants derived from material into which the oligonucleotides have been incorporated are resistant, more resistant or tolerant to the herbicide, when compared to plants derived from material not containing the said oligonucleotide.

25

Example 2 This Example demonstrates the production of *Arabidopsis thaliana* which is resistant to the herbicide glyphosate (and suitable salts thereof). The following oligonucleotides (depicted as SEQ ID Nos 12 to 16 in the sequence listing) are prepared using standard technology.

- 13 -

T to I

*

```

      T GCGCG cauuacguccTTATCguuacgcagg T
5    T                                     T
      T                                     T
      T GCGCG GTAATGCAGGAATAGCAATGCGTCC T
          3'5'      (SEQ ID No. 12)

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10 T to I 2

*

```

      T GCGCG cauuacgtccTTATCguuacgcaag T
      T                                     T
      T                                     T
15    T GCGCG GTAATGCAGGAATAGCAATGCCCTTC T
          3'5'      (SEQ ID No. 13)

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P to S

*

```

      T GCGCG ugucguuacgCAAGTgaauggcgac T
      T                                     T
      T                                     T
25    T GCGCG ACAGCAATGCGTTCACCTACCGCTG T
          3'5'      (SEQ ID No. 14)

```

P to S 2

*

```

      T GCGCG uaucguuacgCAAGTgaauggcgac T
      T                                     T
      T                                     T
30    T GCGCG ATAGCAATGCGTTCACCTACCGCTG T
          3'5'      (SEQ ID No. 15)

```

35

* *

```

      T GCGCG cauuacguccTTATCguuacgCAAGTgaauggcgac T
40    T                                     T
      T                                     T
      T GCGCG GTAATGCAGGAATAGCAATGCGTTCACCTACCGCTG T
          3'5'      (SEQ ID No. 16)

```

45 These oligonucleotides are introduced into *Arabidopsis* by microprojectile bombardment or

bombardments

procedure as described by Seki *et al.* (1991) Appl. Microbiol. Biotechnol. 36 228-230).

Arabidopsis thaliana genotype C24 seeds are surface sterilised and sown on B-5 medium

(Gamborg *et al.*, 1968) solidified with 0.6 % agarose. The plants are grown aseptically for 4-6 weeks under 16 h light 8 h dark at 26 °C. Roots are harvested and cut into sections that are 0.5 - 1.0 cm long and placed onto a filter paper on medium containing B5 salts and vitamins, 3 % sucrose, 0.5 mg/ml 2,4-dichlorophenoxyacetic acid, 0.05 mg/l kinetin and 0.8 % agarose (0.5 - 0.05 medium). After two to five days the roots are ready for bombardment. Gold particles (10 mg; Hereus, 0.4-1.2 µm diameter) are coated with 1 - 100 µg of oligonucleotide as follows. The oligonucleotides may be co-incubated with plasmids comprising sequences encoding proteins capable of forming recombinase complexes in plant cells such that recombination is catalysed between the oligonucleotide and the target sequence. The particles are suspended in 1 ml of absolute ethanol and incubated for three hours at room temperature then stored at -20°C. Twenty to thirty-five µl of sterile resuspended particles are collected by centrifugation in a microcentrifuge. The particles are washed with one ml of sterile distilled water and re-collected by centrifugation. Microprojectiles are then resuspended in 30 µl oligonucleotide solution (1 -100 µg), 25 µl of 1M CaCl₂ is added followed by 10 µl of 0.1 M spermidine (free base). The mixture is incubated on ice for 10 minutes. 1 -10 µl of this solution is used per bombardment. A suitable mixture or combination of oligonucleotides is introduced into plant material either simultaneously or sequentially. If the oligonucleotides are introduced sequentially, they must be introduced in such a way that the mutation governed by the first oligonucleotide is not negated by the mutation governed by a subsequently introduced oligonucleotide. For example, if the oligonucleotide depicted by SEQ ID No. 12 is introduced first, the oligonucleotide depicted by SEQ ID No. 15 should be used subsequently. Alternatively, a single oligonucleotide comprising regions providing for multiple mutations (such as that depicted in SEQ ID No. 16) may be used.

The roots are bombarded with oligonucleotide-coated particles by a helium-driven biolistics PDS 1000 system (BioRad) with a 300 mm Hg vacuum. The levels between the rupture disk and the macrocarrier and the macro-carrier and sample are varied for maximal transformation efficiency. Rupture disks of between 1000 and 2000 psi are used. Two suitable oligonucleotides are introduced into *Arabidopsis* plant material either simultaneously or sequentially. For simultaneous transformation the oligonucleotides are used in equal molar concentrations and may be introduced into the material by multiple firings into the same tissue. For sequential transformation the roots receive at least one

- 15 -

bombardment with each oligonucleotide but multiple firings of each oligonucleotide are used if necessary to optimise transformation efficiencies.

After the bombardments the plant material is transferred to 0.5 - 0.05 medium and incubated at 26°C for one to 5 days. Regeneration of transformed material into *Arabidopsis* plants is performed as Seki *et al* 1991 with the exception that kanamycin or gentamycin are not included in any of the media. Instead the transformed material is selected by its resistance or tolerance to glyphosate, present in the selection medium at a concentration sufficient to kill control material which has been subjected to a like transformation procedure with the *proviso* that it does not contain the oligonucleotides specified above.

DNA uptake by protoplasts incubated in PEG The protocol of Dam *et al.* (1989 Mol Gen. Genet 217 6-12) is followed. Instead of using linearised plasmid DNA in the transformation an equal molar ratio mix of the two oligonucleotides (SEQ ID Nos 12 and 15) are used (1- 100 µg) with 50 -100 µg calf thymus carrier DNA. The oligonucleotides may be co-incubated with plasmids comprising sequences encoding proteins capable of forming recombinase complexes in plant cells such that recombination is catalysed between the oligonucleotide and the target sequence. Glyphosate selection instead of hygromycin selection is applied at the same stage during callus formation. The concentration of glyphosate used is varied to give optimum selection of transformed *Arabidopsis* plants, but is determined by reference to suitable control experiments.

Plants derived from material into which the oligonucleotides have been incorporated are resistant, more resistant or tolerant to the herbicide, when compared to plants derived from material not containing the said oligonucleotide.

Example 3 This Example demonstrates the provision of glyphosate resistant *Brassica napus*

T to I

*

T GCGCG cccuacguccTTATCgouacccagg T

- 16 -

T to I 2

*

```

      T GCGCG ccuacgtccTTATCgcuacgcaag T
T
5  T
      T GCGCG GGAATGCAGGAATAGCCATGCGTTC T
      3'5'      (SEQ ID No. 18)

```

10 P to S

*

```

      T GCGCG uguccgguacgCAAGTgaguggcgac T
T
      T
15 T GCGCG ACAGCCATGCGTTCACTCACCGCTG T
      3'5'      (SEQ ID No. 19)

```

20 P to S 2

*

```

      T GCGCG uauccgguacgCAAGTgaguggcgac T
T
      T
25 T GCGCG ATAGCCATGCGTTCACTCACCGCTG T
      3'5'      (SEQ ID No. 20)

```

* *

```

30 T GCGCG ccuacguccTTATCgcuacgCAAGTgaguggcgac T
T
T
      T GCGCG GGAATGCAGGAATAGCCATGCGTTCACTCACCGCTG T
      3'5'      (SEQ ID No. 21)

```

35 These oligonucleotides are designed to target the *Brassica napus* EPSPS gene. The oligonucleotides provide for two changes in the sequence of the protein encoded by the gene, viz. at T102 and P106 of the Brassica mature enzyme such that the mutant gene (via an altered protein product) confers resistance to glyphosate.

40 The oligonucleotides are introduced into *Brassica napus* using known methods which includes microprojectile bombardment or uptake of DNA by protoplasts.

Bombardments Seeds of *B.napus cv Westar* are surface sterilised in 1% sodium hypochlorite for 20 minutes. The seeds are then washed in sterile water three times and planted at a density of about 10 seeds per plate on Murashige Skoog (MS) minimal organics
 45 medium (GibcoBrl) with 3% sucrose and 0.7% phytagar (Gibco) pH 5.8. Seeds are germinated at 24 °C in 16 h light/8h dark. After five days the cotyledons are excised in such a

way that they include approximately 2 mm of petiole at the base. Care is taken to exclude the apical meristem. The excised cotyledons are placed on MS medium, 3 % sucrose and 0.7 % phytagar enriched with 20 μ M bezyladenine with the petioles imbedded to a depth of 2 mm in the medium at a density of about ten cotyledons per plate.

Gold particles (10 mg; Hereus, 0.4-1.2 μ m diameter) are coated with 1 - 100 μ g of oligonucleotide (SEQ ID No. 22 for example, or SEQ ID Nos. 18 and 20) in plant cells. The oligonucleotides may be co-incubated with plasmids comprising sequences encoding proteins capable of forming recombinase complexes in plant cells such that recombination is catalysed between the oligonucleotide and the target sequence. The particles are suspended in 1 ml of absolute ethanol and incubated for three hours at room temperature then stored at -20°C. Twenty to thirty five μ l of sterile resuspended particles are collected by centrifugation in a microcentrifuge. The particles are washed with one ml of sterile distilled water and re-collected by centrifugation. Microprojectiles are then resuspended in 30 μ l solution (containing oligonucleotides depicted in SEQ ID Nos. 18 and 20, for example in an amount of about 1 -100 μ g). 25 μ l of 1M CaCl_2 is added followed by 10 μ l of 0.1 M spermidine (free base). The mixture is incubated on ice for 10 minutes. 1 -10 μ l of this solution is used per bombardment.

The cotyledons are bombarded with oligonucleotide-coated particles by a helium-driven biolistics PDS 1000 system (BioRad) with a 300 mm Hg vacuum. The levels between the rupture disk and the macrocarrier and the macro-carrier and sample are varied for maximal transformation efficiency. Rupture disks of between 1000 and 2000 psi are used. The two oligonucleotides are introduced into the *Brassica* plant material either simultaneously or sequentially. For simultaneous transformation the oligonucleotides are used in equal molar concentrations and may be introduced into the explant by multiple firings into the same tissue. For sequential transformation the explants receive at least one bombardment with each oligonucleotide but multiple firings of each oligonucleotide are used as necessary to optimise transformation efficiencies.

After bombardment the explants are placed onto regeneration medium comprising

including selective concentrations of glyphosate. The petioles remain embedded in the media. The explants are left for 2–6 weeks, and then transferred onto MS medium.

- 18 -

supplemented with 3 % sucrose, 0.7% phytagar pH 5.8 and selecting concentrations of glyphosate. One to three weeks later surviving shoots are transferred to rooting media which comprises MS medium, 3% sucrose, 2 mg/ml indole butyric acid, 0.7% phytagar with no glyphosate. Once roots are visible the plants are transferred to pots and propagated in the greenhouse.

Protoplast uptake The method of Golz *et al.* ((1990) Plant Mol Biol 15 475 - 483) is followed. *Brassica napus* genotype H1 is used. Instead of using plasmid DNA in the transformation an equal molar ratio mix of the two oligonucleotides (SEQ ID Nos 18 and 20) are used (1- 100 µg) and 20 -100 µg calf thymus carrier DNA. The oligonucleotides may be co-incubated with plasmids comprising sequences encoding proteins capable of forming recombinase complexes in plant cells such that recombination is catalysed between the oligonucleotide and the target sequence. Glyphosate selection instead of hygromycin selection is applied at the same stage during callus formation. The concentration of glyphosate used is varied to give optimum selection of transformed *Brassica* plants.

Plants derived from material into which the oligonucleotides have been incorporated are resistant, more resistant or tolerant to the herbicide, when compared to plants derived from material not containing the said oligonucleotide.

Example 4 This Example demonstrates the provision of corn resistant to the herbicide glyphosate (and salts thereof).

T to I

T GCGCG ccuacgaccTTAGCGuuacgccggu T
T T T
T CGCGC GGAATGCTGGAATCGCAATGCGGCCAT T
3'5' (SEQ ID No. 22)

T GCGCG ccuacgaccTTAGCGuuacgccagua T
T T T
T CGCGC GGAATGCTGGAATCGCAATGCGGTCAT T
3'5' (SEQ ID No. 23)

- 19 -

P to S

*

T GCGCG gaaguuacgCCAGTaacugucgucg T
 T T T
 5 T T T
 T CGCGC CTGCAATGCGGTCATTGACAGCAGC T
 3'5' (SEQ ID No. 24)

P to S 2

*

10 T GCGCG agcguuacgCCAGTaacugtcgucg T
 T T T
 T T T
 T CGCGC TCGCAATGCGGTCATTGACAGCAGC T
 15 3'5' (SEQ ID No. 25)

**

*

20 T GCGCG ccuuacgaccTTAGCGuuacgCCAGTaacugucgucg T
 T T T
 T T T
 T CGCGC GGAATGCTGGAATCGCAATGCGGTCATTGACAGCAGC T
 3'5' (SEQ ID No. 26)

25 These oligonucleotides which are designated as SEQ ID Nos 22-26 in the sequence listing and which are produced by means known to the skilled man, may be introduced into corn using silicon carbide whiskers, pollen harbouring oligonucleotides or via pollen tubes.

Silicon carbide whiskers This transformation is performed essentially as described by Frame *et al.* (1994 Plant J. 6 941-948). The oligonucleotide depicted as SEQ ID No 26 (1-
 30 100 µg) is added to the whiskers and used to transform A188 x B73 cell suspensions. The oligonucleotides may be co-incubated with plasmids comprising sequences encoding proteins capable of forming recombinase complexes in plant cells such that recombination is catalysed between the oligonucleotide and the target sequence. Plant regeneration is performed using selective concentrations of glyphosate in place of bialophos. Plants are
 35 transferred to pots and are then matured in the green house. Caryopsis from these plants are germinated in soil and sprayed with a selecting concentration of glyphosate 9 to 14 days post emergence.

40 ID Nos 23 and 25). The oligonucleotides may be co-incubated with plasmids comprising sequences encoding proteins capable of forming recombinase complexes in plant cells such

that recombination is catalysed between the oligonucleotide and the target sequence.

Bombarded pollen is applied to receptive silks of detassled plants. Sufficient replicas are performed to pollinate a large number (typically up to 300) of plants. Progeny of the plants are screened for glyphosate resistant members of the population by spraying with selecting concentrations of glyphosate.

Pollen tube mediated transformation Emasculated corn plants are used. Wild type pollen is applied to pollination receptive silks. After between 30 min to 6 hours the silks are cut to within one cm of the base. Suitable mixtures of the above oligonucleotides (1-100µg/10 µl in TE) are applied to the cut surface using a 1 ml syringe and needle such that surface is completely covered. The oligonucleotides may be co-incubated with plasmids comprising sequences encoding proteins capable of forming recombinase complexes in plant cells such that recombination is catalysed between the oligonucleotide and the target sequence. The plants are then grown in a green house with an initial humidity of about 75 %. Progeny of the plants are screened for glyphosate resistant members of the population by spraying with selecting concentrations of glyphosate.

Plants derived from material into which the oligonucleotides have been incorporated are resistant, more resistant or tolerant to the herbicide, when compared to plants derived from material not containing the said oligonucleotide.

Example 5 This Example demonstrates the provision of tomato plants resistant to a bleaching herbicide designated as R390244.

*

```

T GCGCC agcguaacuuGTCGAaagaagucca T
T                                     T
T                                     T
25 T CGCGC TCGCATTGAACAGCTTTCTTCAGGT T
      3' 5'      (SEQ ID No. 27)
  
```

This oligonucleotide (SEQ ID No. 27) is designed to target the codon for arginine 307 of the tomato phytoene desaturase (PDS) gene and introduce a mutation such that the mutant PDS is resistant to the herbicide R390244. The oligonucleotides may be co-incubated with plasmids comprising sequences encoding proteins capable of forming recombinase complexes in plant cells such that recombination is catalysed between the oligonucleotide and the target sequence. The oligonucleotide is introduced into tomato Mill cv H722 via microprojectile bombardment essentially as described by Eck *et al.* (1995 Plant Cell Reports

14, 299-304) and as outlined above for the other crops subjected to this transformation procedure.

Regenerable cotyledon explant material (as described by Fillati *et al.* (1997 Bio/technology 5 726-730) suspensions are bombarded with SEQ ID No. C oligonucleotide-coated particles by a helium-driven biolistics PDS 1000 system (BioRad) with a 300 mm Hg vacuum. The levels between the rupture disk and the macrocarrier and the macro-carrier and sample are varied for maximal transformation efficiency. Rupture disks of between 1000 and 2000 psi are used. The oligonucleotide may be introduced into the explant by multiple firings into the same tissue as necessary to optimise transformation efficiencies. The
10 regenerable cotyledons are bombarded at the same stage as when *Agrobacterium* is used in the method of Beaudoin and Rothstein (1997 Plant Mol Biol 33 835 -846). Regeneration of tomato plants is as described by Beaudoin and Rothstein except that no selection agent is used. Primary putative transformants are grown in the greenhouse and cuttings are propagated in soil. These cuttings, once established, are sprayed with selecting
15 concentrations of R390244 and allow transformed herbicide resistant plants to be identified. These transformed plants are grown to maturity and seeds resulting from self pollination are collected.

Mutation events in individuals is confirmed by amplifying the particular mutant gene sequence from herbicide resistant individuals spanning the region of mutation by PCR and
20 sequencing individually isolated and cloned sequences.

Plants derived from material into which the oligonucleotides have been incorporated are resistant, more resistant or tolerant to the herbicide, when compared to plants derived from material not containing the said oligonucleotide.

- 22 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

5 (i) APPLICANT:

(A) NAME: ZENECA LTD
 (B) STREET: 15 Stanhope Gate
 (C) CITY: LONDON
 (E) COUNTRY: GB
 10 (F) POSTAL CODE (ZIP): W1Y 6LN

(ii) TITLE OF INVENTION: IMPROVEMENTS IN OR RELATING TO ORGANIC COMPOUNDS

15 (iii) NUMBER OF SEQUENCES: 29

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
 (B) COMPUTER: IBM PC compatible
 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 20 (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

25 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1944 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 30 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

35 (iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Petunia hybrida

40 (ix) FEATURE:

(A) NAME/KEY: CDS
 (B) LOCATION: 28..1578

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GAATTCCTC AATCTTTACT TTCAAGA ATG GCA CAA ATT AAC AAC ATG GCT	51
Met Ala Gln Ile Asn Asn Met Ala	
1 5	
50 CAA GGG ATA CAA ACC CTT AAT CCC AAT TCC AAT TTC CAT AAA CCC CAA	99
Gln Gly Ile Gln Thr Leu Asn Pro Asn Ser Asn Phe His Lys Pro Gln	
10 15 20	
55 GTT CCT AAA TCT TCA AGT TTT CTT GTT TTT GGA TCT AAA AAA CTG AAA	147
Val Pro Lys Ser Ser Ser Phe Leu Val Phe Gly Ser Lys Lys Leu Lys	
25 30 35 40	
AAT TCA GCA AAT TCT ATG TTG GTT TTG AAA AAA GAT TCA ATT TTT ATG	195
Asn Ser Ala Asn Ser Met Leu Val Leu Lys Lys Asp Ser Ile Phe Met	
45 50 55	
CAA AAG TTT TGT TCC TTT AGG ATT TCA GCA TCA GTG GCT ACA GCA CAG	243
Gln Lys Phe Cys Ser Phe Arg Ile Ser Ala Ser Val Ala Thr Ala Gln	
60 65 70	

- 23 -

	AAG CCT TCT GAG ATA GTG TTG CAA CCC ATT AAA GAG ATT TCA GGC ACT	791
	Lys Pro Ser Glu Ile Val Leu Gln Pro Ile Lys Glu Ile Ser Gly Thr	
	75 80 85	
5	GTT AAA TTG CCT GGC TCT AAA TCA TTA TCT AAT AGA ATT CTC DTT CTT	339
	Val Lys Leu Pro Gly Ser Lys Ser Leu Ser Asn Arg Ile Leu Leu Leu	
	90 95 100	
10	GCT GCC TTA TCT GAA GGA ACA AAT GTG GGT CAC AAT TTA CTA AGT AGT	387
	Ala Ala Leu Ser Glu Gly Thr Thr Val Val Asp Asn Leu Leu Ser Ser	
	105 110 115 120	
15	GAT GAT ATT CAT TAC ATG CTT GGT GGC TTG AAA ACA CTT GGA CTG CAT	435
	Asp Asp Ile His Tyr Met Leu Gly Ala Leu Lys Thr Leu Gly Leu His	
	125 130 135	
20	GTA GAA GAA GAT AGT GCA AAC CAA CGA GGT GTT GTT GAA GGT TGT GGT	483
	Val Glu Glu Asp Ser Ala Asn Gln Arg Ala Val Val Glu Gly Cys Gly	
	140 145 150	
	GGG CTT TTC CCT GTT GGT AAA GAG TCC AAG GAA GAA ATT CAA CTG TTC	531
	Gly Leu Phe Pro Val Gly Lys Glu Ser Lys Glu Glu Ile Gln Leu Phe	
	155 160 165	
25	CTT GGA AAT GCA GGA ACA GCA ATG CGG CCA CTA ACA GCA GCA GTT ACT	579
	Leu Gly Asn Ala Gly Thr Ala Met Arg Pro Leu Thr Ala Ala Val Thr	
	170 175 180	
30	GTA GCT GGT GGA AAT TCA AGG TAT GTA CTT GAT GGA GTT CCT CGA ATG	627
	Val Ala Gly Gly Asn Ser Arg Tyr Val Leu Asp Gly Val Pro Arg Met	
	185 190 195 200	
35	AGA GAG AGA CCA ATT AGT GAT TTG GTT GAT GGT CTT AAA CAG CTT GGT	675
	Arg Glu Arg Pro Ile Ser Asp Leu Val Asp Gly Leu Lys Gln Leu Gly	
	205 210 215	
40	GCA GAG GTT GAT TGT TTC CTT GGT ACG AAA TGT CCT CCT GTT CGA ATT	723
	Ala Glu Val Asp Cys Phe Leu Gly Thr Lys Cys Pro Pro Val Arg Ile	
	220 225 230	
	GTC AGC AAG GGA GGT CTT CCT GGA GGG AAG GTC AAG CTC TCT GGA TCC	771
	Val Ser Lys Gly Gly Leu Pro Gly Gly Lys Val Lys Leu Ser Gly Ser	
	235 240 245	
45	ATT AGC ACC CAA TAC TTG ACT GGT CTG CTT ATG GGT GGT CCA CTG GGT	819
	Ile Ser Ser Gln Tyr Leu Thr Ala Leu Leu Met Ala Ala Pro Leu Ala	
	250 255 260	
50	TTA CGA GAT GTG GAG ATT GAA ATC ATT GAG AAA CTA ATT AGT GTA CCG	867
	Leu Gly Asp Val Glu Ile Glu Ile Ile Asp Lys Leu Ile Ser Val Pro	
	265 270 275 280	
55	TAT GTC GAG ATG ACA TTG AAG TTG ATG GAG CGA TTT GGT ATT TCT GTG	915
	Tyr Val Glu Met Thr Leu Lys Leu Met Glu Arg Phe Gly Ile Ser Val	
	285 290 295	
	TAT AAG TCT GAT GAA AAA GAT TTT CTT CAA GAT TTT TCA AAT GAT	963
	Tyr Lys Ser Phe Gly Lys Ala Phe Val Gln Gly Asp Ala Ser Ser Ala	
	315 320 325	

- 24 -

	AGC TAC TTC TTG GCT GGT GCA GCA GTC ACA GGT GGA ACT ATC ACT GTT	1059
	Ser Tyr Phe Leu Ala Gly Ala Ala Val Thr Gly Gly Thr Ile Thr Val	
	330 335 340	
5	GAA GGT TGT GGG ACA AAC AGT TTA CAG GGG GAT GTC AAA TTT GCT GAG	1107
	Glu Gly Cys Gly Thr Asn Ser Leu Gln Gly Asp Val Lys Phe Ala Glu	
	345 350 355 360	
10	GTA CTT GAA AAA ATG GGA GCT GAA GTT ACG TGG ACA GAG AAC AGT GTC	1155
	Val Leu Glu Lys Met Gly Ala Glu Val Thr Trp Thr Glu Asn Ser Val	
	365 370 375	
15	ACA GTC AAA GGA CCT CCA AGG AGT TCT TCT GGG AGG AAG CAT TTG CGT	1203
	Thr Val Lys Gly Pro Pro Arg Ser Ser Ser Gly Arg Lys His Leu Arg	
	380 385 390	
	GCC ATT GAT GTG AAC ATG AAT AAA ATG CCT GAT GTT GCC ATG ACA CTT	1251
	Ala Ile Asp Val Asn Met Asn Lys Met Pro Asp Val Ala Met Thr Leu	
	395 400 405	
20	GCT GTT GTT GCA CTT TAT GCT GAT GGT CCC ACA GCT ATA AGA GAT GTT	1299
	Ala Val Val Ala Leu Tyr Ala Asp Gly Pro Thr Ala Ile Arg Asp Val	
	410 415 420	
25	GCT AGC TGG AGA GTC AAG GAA ACT GAG CGC ATG ATC GCC ATA TGC ACA	1347
	Ala Ser Trp Arg Val Lys Glu Thr Glu Arg Met Ile Ala Ile Cys Thr	
	425 430 435 440	
30	GAA CTT AGG AAG TTA GGA GCA ACC GTT GAA GAA GGA CCA GAC TAC TGC	1395
	Glu Leu Arg Lys Leu Gly Ala Thr Val Glu Glu Gly Pro Asp Tyr Cys	
	445 450 455	
35	ATA ATC ACC CCA CCG GAG AAA CTA AAT GTG ACC GAT ATT GAT ACA TAC	1443
	Ile Ile Thr Pro Pro Glu Lys Leu Asn Val Thr Asp Ile Asp Thr Tyr	
	460 465 470	
	GAT GAT CAC AGG ATG GCC ATG GCT TTT TCT CTT GCT GCT TGT GCA GAT	1491
	Asp Asp His Arg Met Ala Met Ala Phe Ser Leu Ala Ala Cys Ala Asp	
	475 480 485	
40	GTT CCC GTC ACC ATC AAT GAC CCT GGC TGC ACG CGG AAA ACC TTC CCT	1539
	Val Pro Val Thr Ile Asn Asp Pro Gly Cys Thr Arg Lys Thr Phe Pro	
	490 495 500	
45	AAC TAC TTT GAT GTA CTT CAG CAG TAC TCC AAG CAT TGA ACCGCTTCCC	1588
	Asn Tyr Phe Asp Val Leu Gln Gln Tyr Ser Lys His	
	505 510 515	
50	TATATTGCAG AATGTAAGTA AGAATATGTG AAGAGTTTAG TTCTTGTACA AGACAGGCTA	1648
	CGACTGCCTG GTATCAGAAC CACAATGGGT TCCATTTTCA TTCAGAAGGG CATTCCAAGG	1708
	CTTCGAACTC TTTACTTATT TGCGAGTGAT GAAATGTATT TGTTAGAGTT GAGCTTCTTT	1768
55	TTGTCTTTAA GGAATGTACA CTAATAGAGT TAAGAATTAC TAGTATGGGC CAGTGTAAGG	1828
	AGTACTATTA CTCTTTGCTT ATTTTATGTA TTGAGTTTTG TCAAGGATCT GGCTTTGTCA	1888
60	AGAATTACTG GTTAATTTTA TTGACAATCT CATGTGTCTA AATGAAATTG TTTGAT	1944

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 517 amino acids

65

- 25 -

(B) TYPE: amine acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Ala Gln Ile Asn Asn Met Ala Gln Gly Ile Gln Thr Leu Asn Pro
 1 5 10 15
 10 Asn Ser Asn Phe His Lys Pro Gln Val Pro Lys Ser Ser Ser Phe Leu
 20 25 30
 Val Phe Gly Ser Lys Lys Leu Lys Asn Ser Ala Asn Ser Met Leu Val
 35 40 45
 15 Leu Lys Lys Asp Ser Ile Phe Met Gln Lys Phe Cys Ser Phe Arg Ile
 50 55 60
 Ser Ala Ser Val Ala Thr Ala Gln Lys Pro Ser Glu Ile Val Leu Gln
 20 65 70 75 80
 Pro Ile Lys Glu Ile Ser Gly Thr Val Lys Leu Pro Gly Ser Lys Ser
 85 90 95
 25 Leu Ser Asn Arg Ile Leu Leu Leu Ala Ala Leu Ser Glu Gly Thr Thr
 100 105 110
 Val Val Asp Asn Leu Leu Ser Ser Asp Asp Ile His Tyr Met Leu Gly
 115 120 125
 30 Ala Leu Lys Thr Leu Gly Leu His Val Glu Glu Asp Ser Ala Asn Gln
 130 135 140
 Arg Ala Val Val Glu Gly Cys Gly Gly Leu Phe Pro Val Gly Lys Glu
 35 145 150 155 160
 Ser Lys Glu Glu Ile Gln Leu Phe Leu Gly Asn Ala Gly Thr Ala Met
 165 170 175
 40 Arg Pro Leu Thr Ala Ala Val Thr Val Ala Gly Gly Asn Ser Arg Tyr
 180 185 190
 Val Leu Asp Gly Val Pro Arg Met Arg Glu Arg Pro Ile Ser Asp Leu
 195 200 205
 45 Val Asp Gly Leu Lys Gln Leu Gly Ala Glu Val Asp Cys Phe Leu Gly
 210 215 220
 Thr Lys Cys Pro Pro Val Arg Ile Val Ser Lys Gly Gly Leu Pro Gly
 50 225 230 235 240
 Gly Lys Val Lys Leu Ser Gly Ser Ile Ser Ser Gln Tyr Leu Thr Ala
 245 250 255
 55 Leu Leu Met Ala Ala Pro Leu Ala Leu Gly Asp Val Gln Ile Glu Ile
 260 265 270
 Phe Phe Val Arg Gly Gly Glu Lys Tyr Lys Ser Pro Gly Lys Ala Phe
 275 280 285 290 295

- 26 -

Val Glu Gly Asp Ala Ser Ser Ala Ser Tyr Phe Leu Ala Gly Ala Ala
 325 330 335

5 Val Thr Gly Gly Thr Ile Thr Val Glu Gly Cys Gly Thr Asn Ser Leu
 340 345 350

Gln Gly Asp Val Lys Phe Ala Glu Val Leu Glu Lys Met Gly Ala Glu
 355 360 365

10 Val Thr Trp Thr Glu Asn Ser Val Thr Val Lys Gly Pro Pro Arg Ser
 370 375 380

Ser Ser Gly Arg Lys His Leu Arg Ala Ile Asp Val Asn Met Asn Lys
 385 390 395 400

Met Pro Asp Val Ala Met Thr Leu Ala Val Val Ala Leu Tyr Ala Asp
 405 410 415

20 Gly Pro Thr Ala Ile Arg Asp Val Ala Ser Trp Arg Val Lys Glu Thr
 420 425 430

Glu Arg Met Ile Ala Ile Cys Thr Glu Leu Arg Lys Leu Gly Ala Thr
 435 440 445

25 Val Glu Glu Gly Pro Asp Tyr Cys Ile Ile Thr Pro Pro Glu Lys Leu
 450 455 460

Asn Val Thr Asp Ile Asp Thr Tyr Asp Asp His Arg Met Ala Met Ala
 465 470 475 480

Phe Ser Leu Ala Ala Cys Ala Asp Val Pro Val Thr Ile Asn Asp Pro
 485 490 495

35 Gly Cys Thr Arg Lys Thr Phe Pro Asn Tyr Phe Asp Val Leu Gln Gln
 500 505 510

Tyr Ser Lys His
 515

40 (2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 12 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: unknown

45 (ii) MOLECULE TYPE: peptide

50 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

55 (vi) ORIGINAL SOURCE:
 (A) ORGANISM: Brassica napus

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

60 Leu Gly Asn Ala Gly Thr Ala Met Arg Pro Leu Thr
 1 5 10

65 (2) INFORMATION FOR SEQ ID NO: 4:

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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown
(ii) MOLECULE TYPE: peptide
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(vi) ORIGINAL SOURCE:
(A) ORGANISM: Brassica napus
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:
Met Ala Ala Pro Leu Ala Leu Gly Asp Val Glu Ile
1 5 10

(2) INFORMATION FOR SEQ ID NO: 5:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown
(ii) MOLECULE TYPE: other nucleic acid
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(vi) ORIGINAL SOURCE:
(A) ORGANISM: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:
ATTGAGTGTG AACTTGGGAA TGCAGGAGCA GGCATCGGTC CACTCAGGCT TGGT

(3) INFORMATION FOR SEQ ID NO: 6:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown
(ii) MOLECULE TYPE: other nucleic acid

(A) ORGANISM: synthetic

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

5 GAGUGGACGC AUGGCUETTG CTGCAUUCOC AAGGUACAA 33

(2) INFORMATION FOR SEQ ID NO: 7:

10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

15 (ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

20 (iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: synthetic

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

ACTGCCCTCC TCATGGCAGC TCCTTTAGCT CTTGGAGACG TGGAGATTGA GATCATT 57

30 (2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
35 (B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid

40 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

45 (vi) ORIGINAL SOURCE:
(A) ORGANISM: synthetic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

50 AAUCUCCACG UCUGCAAGAG TTAAAGGAGC UGCCAUGAGG A 41

(2) INFORMATION FOR SEQ ID NO: 9

55 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3831 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

60 (ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

65 (iv) ANTI-SENSE: NO

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(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Brassica napus*

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

5 AGATCTTAAA GGCTCTTTTC CAGTCTCAGT TACCAAAACT ATAAGAAAAT CCACTTGCTG 60
 TGTGAAATAG TCGACGTGCA TAAAGTACTT AAGACGTGGC ACATTATTAT TGGCTACTAG 120
 10 AAAAAAAACT CATACACCAT CGTAGAGGT GGGGTGGTG AAGAATTTGA TGGGTGGCTC 180
 TCCCCCCCCC ACTCAGTAAA CTCATGTTCT TTGTAAGGCG CTCACTACAA CAACAAAGGA 240
 GAGGACAGTG CTATAGAAAA GCTTTCAAAT TCAATCAATG CCGCATCTCA GCAGAATCTG 300
 15 CCAATGGCGTG CAGAACCCAT GTGTTATCAT CTCCAATCTC TCCAAATCCA ACCAAAACAA 360
 ATCACCTTTC TCGCTCTCCT TGAAGACGCA TCAGCTTGA GCTTCTTCGT GGGGATTGAA 420
 20 GAAGAGTGGA AGGATGCTAA ACGGTTCTGT AATTCGCCCG GTTAAGGTAA CAGCTTCTGT 480
 TTCCACGTCC GAGAAAGCTT CAGAGATTGT GCTTCAACCA ATCAGAGAAA TCTCGGCTCT 540
 CATTAAGCTA CCGCGATCCA AATCTCTCTC CAATCGGATC CTCCTTCTTG CCGCTCTATC 600
 25 TGAGGTACAT ATACTTGCTT AGTGTTAGGC CTTTGTGTG AGATTTTGGG AACTATAGAC 660
 AATTTAGTAA GAATTTATAT ATAATTTTTT TAAAAAAAT CAGAAGCCTA TATATATTTA 720
 30 AATTTTTCOA AAATTTTGG AGGTTATAGG CTTATGTTAC ACCATTCTAG TCTGCATCTT 780
 TCGGTTTGAG AATGAAGAAT TTTATTTTTT AAAAATTAT TATAGGGAAC TACTGTAGTG 840
 GACAACCTGT TGAACAGTGA TGACATCAAC TACATCTTG ATGCGTTGAA GAAGCTGGGG 900
 35 CTTAACGTGG AACGTGACAG TGTAACAAC CGTGCGGTTG TTGAAGGATG CCGTGGAATA 960
 TTCCCAGCTT CCTTAGATTC CAAGAGTGAT ATTGAGTTGT ACCTTGGGAA TGCAGGAACA 1020
 40 GCCATGCGTC CACTCACCGC TGCAGTTACA GCTCCAGGTG GCAACGCGAG GTAAAGTTAA 1080
 CGAGTTTTTT GTTATTCTCA AGAAATTGAT CTTGTGTTTG ATGCTTTTAG TTTGTTTGT 1140
 TTTCTAGTTA TGTACTTGAT GGGGTGCCCA GAATGAGGGA AAGAGCTATA GGAGATTTCG 1200
 45 TTGTTGGTGT TARGCAGCTT GGTGCTGATG TTGATGTAG TGTGGCACT AACTGTCTCT 1260
 CTGTTGGTGT CAATGCTAAT GGTGGGCTTC CGGTGGAAA GGTGATCTTC ACATTTACTC 1320
 50 TATGAATTGT TTGCAGCAGT GTTGTTCAT CACAGCCTTT GATTGACATT ATTTGATCTT 1380
 TTAGTTTGT GTTATATTAC TTGATGGATC TTTAAAAGG AATTGGGTCT GSTGTGAAAG 1440
 TGAITAGCAA TCTTCTCGA TTGCTTGAA GGTGTTGGG ATTATTAAT GAGAGATTAG 1500
 55 CCAATTAACT TCGAAATTT TCGAAAAA ATTATTAAT TCGGTAAGA TGGTCTTTTC 1560
 TGTATGAT CATACTTA TTTTAAAG TTTTAAAG TTTTAAAG TTTTAAAG 1620
 GAGAGTTGA GATTGAGAT ATTGATAAG TTTATATCT TGTATATCTT GAAATGAGAT 1680

	TGAAGTTGAT GGAGCGTTTT GGTGTTAGTG CCGAGCATAG TGATAGCTGG GATCGTTTCT	1860
	TTGTCAAGGG CGGTGAGAAA TACAAGTAAT GAGTTCTTTT AAGTTGAGAG TTAGATTGAA	1920
5	GAATGAATGA CTGATTAAAC AAATGGCAAA ACTGATTCAG GTGGCCTGGT AATGCTTATG	1980
	TAGAAGGTGA TGCTTCTAGT GCTAGCTATT TCTTGGGTGG TGCTGCCATT ACTGCTGAAA	2040
10	CTGTTACTGT CGAAGGTTCT GGAACAATA GCGTCCAGGT AGTTTATCCA CTCTGAATCA	2100
	TCAAATATTA TTCTCCTCTC GTTTTATGTT AAGTGTCAAT AGCTTTTAAA TTTTGTTC	2160
	TTAAAAGTGT CATTTTACAT TTTCAATGCA TATATTAAAT AAATTTTCCA GTTTTACTA	2220
15	ATTCATTAAT TAGCAAAATC AAACAAAAT TATATTAAAT AATGTAAAT TCGTAATTTG	2280
	TGTGCAATA CCTTAAACCT TATGAAGGG AAACCTTATG AAACAGAGGG AGTACTAATT	2340
20	TTATAATAAA ATTTGATTAG TTCAAAGTGG TGTATAACAT GTTTTGTAG AATCTAAGCT	2400
	CATTCTCTTT TTATTTTTTG TGATGAATCC AAAGGGAGAT GTGAAATTCG CAGAGGTTCT	2460
	TGAGAAAATG CCACTGTAAG TGTGATGGAG AGAGAACACT GTGACTGTGA CTGGACCATC	2520
25	AAGAGATGCT TTTGGAATGA GSCACTTGGG TGCTGTTGAT GTCAACATGA ACAAATGCC	2580
	TGATGTAGCC ATGACTCTAG CCGTTGTTGC TCTCTTTGCC GATGGTCCAA CCACCATCAG	2640
30	AGATGGTAAA GCAAAACCCT CTCTTTGAAT CAGCGTGTTC TAAAAGATTC ATGGTTGCTT	2700
	AAACTCTATT TGGTCAATGT AGTGGCTAGC TGGAGAGTTA AGGAGACAGA GAGGATGATT	2760
	GCCATTTGCA CAGAGCTTAG AAAGGTAAGT TTCCTTTTCT CTCATGCTCT CTCATTGCAA	2820
35	GTTAATCGTT GCATAACTTT TTGCGGTTTT TTTTTTGCG TTCAGCTGG AGCTACAGTG	2880
	GAAGAAGGTT CAGATTATTG TGTGATAACT CCACCAGCAA AGGTGAAACC GCGGAGATT	2940
40	GATACGTATG ATGATCATAG AATGGCGATG GCGTTCTCGC TTGCAGCTTG TGCTGATGTT	3000
	CCAGTCACCA TCAAGGATCC TGGCTGCACC AGGAAGACTT TCGCTGACTA CTTCCAAGTC	3060
	CTTGAAAGTA TCACAAAGCA TTAAAAGACC CTTTCCTCTG ATCCAAATGT GAGAACTGT	3120
45	TGCTTTCTCT TTGTTGCCAC TGTAACATTT ATTAGAAGAA CAAAGTGTGT GTGTTAAGAG	3180
	TGTGTTTGCT TGTAAATGAAC TGASTGAGAT GCAATCGTTG AATCAGTTTT GGGCCTTAAT	3240
50	AAAGGGTTTA GGAAGCTGCA GCGAGATGAT TGTTTTTGAT CGATCATCTT TGAAAATGTG	3300
	TTTGTTTGAG TAATTTTTCT AGGGTTGAGT TGATTACACT AAGAAACACT TTTTGATTTT	3360
	CTATTACACC TATAGACACT TCTTACATGT GACACACTTT GTTGTGGCA AGCAACAGAT	3420
55	TGTGGACAAT TTGCGCTTTA ATGGAAAGAA CACAGTTGTG GATGGGTGAT TTGTGGACGA	3480
	TTCCATGTGT GGTAGGGTG ATTTGTGGAC GGATGATGTG TAGATGAGTG ATGAGTAATG	3540
60	TGTGAATATG TGATGTAAAT GTGTTTATAG TAGATAAGTG GACAACTCT CTSTTTTGAT	3600
	TCCATAAAAC TATACAACAA TACGTGGACA TGGACTCATG TTAATAAAAT TATACCGTAA	3660
	AACGTGGACA CGSACTCTGT ATCTCCAATA CAAACACTTG GCTTCTTCAG CTCAATTGAT	3720
65	AAATTATCTG CAGTTAAACT TCAATCAAGA TGAGAAAGAG ATGATATTGT GAATATGAGC	3780

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GGAGAGAGAA ATCGAAGAAG COTTTACCTT TTGTCGGAGA GTAATAGATC T

3831

(2) INFORMATION FOR SEQ ID NO: 10:

5

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 516 amino acids

(B) TYPE: amino acid

10

(C) STRANDEDNESS: single

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

15

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

20

(A) ORGANISM: Brassica napus

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

25

Met Ala Gln Ser Ser Arg Ile Cys His Gly Val Gln Asn Pro Cys Val
1 5 10 15Ile Ile Ser Asn Leu Ser Lys Ser Asn Gln Asn Lys Ser Pro Phe Ser
20 25 30

30

Val Ser Leu Lys Thr His Gln Pro Arg Ala Ser Ser Trp Gly Leu Lys
35 40 45

35

Lys Ser Gly Thr Met Leu Asn Gly Ser Val Ile Arg Pro Val Lys Val
50 55 60Thr Ala Ser Val Ser Thr Ser Glu Lys Ala Ser Glu Ile Val Leu Gln
65 70 75 80

40

Pro Ile Arg Glu Ile Ser Gly Leu Ile Lys Leu Pro Gly Ser Lys Ser
85 90 95Leu Ser Asn Arg Ile Leu Leu Leu Ala Ala Leu Ser Glu Gly Thr Thr
100 105 110

45

Val Val Asp Asn Leu Leu Asn Ser Asp Asp Ile Asn Tyr Met Leu Asp
115 120 125Ala Leu Lys Lys Leu Gly Leu Asn Val Glu Arg Asp Ser Val Asn Asn
130 135 140

50

Arg Ala Val Val Glu Gly Cys Gly Gly Ile Phe Pro Ala Ser Leu Asp
145 150 155 160

55

Ser Lys Ser Asp Ile Glu Leu Tyr Leu Gly Asn Ala Gly Thr Ala Met
165 170 175Val Val Gly Leu Lys Gln Leu Gly Ala Arg Val Glu Lys Thr Leu Gly
210 215 220

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Thr Asn Cys Pro Pro Val Arg Val Asn Ala Asn Gly Gly Leu Pro Gly
 225 230 235 240
 5 Gly Lys Val Lys Leu Ser Gly Ser Ile Ser Ser Gln Tyr Leu Thr Ala
 245 250 255
 Leu Leu Met Ala Ala Pro Leu Ala Leu Gly Asp Val Glu Ile Glu Ile
 260 265 270
 10 Ile Asp Lys Leu Ile Ser Val Pro Tyr Val Glu Met Thr Leu Lys Leu
 275 280 285
 Met Glu Arg Phe Gly Val Ser Ala Glu His Ser Asp Ser Trp Asp Arg
 290 295 300
 15 Phe Phe Val Lys Gly Gly Gln Lys Tyr Lys Ser Pro Gly Asn Ala Tyr
 305 310 315 320
 Val Glu Gly Asp Ala Ser Ser Ala Ser Tyr Phe Leu Ala Gly Ala Ala
 325 330 335
 20 Ile Thr Gly Glu Thr Val Thr Val Glu Gly Cys Gly Thr Thr Ser Leu
 340 345 350
 25 Gln Gly Asp Val Lys Phe Ala Glu Val Leu Glu Lys Met Gly Cys Lys
 355 360 365
 Val Ser Trp Thr Glu Asn Ser Val Thr Val Thr Gly Pro Ser Arg Asp
 370 375 380
 30 Ala Phe Gly Met Arg His Leu Arg Ala Val Asp Val Asn Met Asn Lys
 385 390 395 400
 Met Pro Asp Val Ala Met Thr Leu Ala Val Val Ala Leu Phe Ala Asp
 405 410 415
 35 Gly Pro Thr Thr Ile Arg Asp Val Ala Ser Trp Arg Val Lys Glu Thr
 420 425 430
 40 Glu Arg Met Ile Ala Ile Cys Thr Glu Leu Arg Lys Leu Gly Ala Thr
 435 440 445
 Val Glu Glu Gly Ser Asp Tyr Cys Val Ile Thr Pro Pro Ala Lys Val
 450 455 460
 45 Lys Pro Ala Glu Ile Asp Thr Tyr Asp Asp His Arg Met Ala Met Ala
 465 470 475 480
 50 Phe Ser Leu Ala Ala Cys Ala Asp Val Pro Val Thr Ile Lys Asp Pro
 485 490 495
 Gly Cys Thr Arg Lys Thr Phe Pro Asp Tyr Phe Gln Val Leu Glu Ser
 500 505 510
 55 Ile Thr Lys His
 515

(2) INFORMATION FOR SEQ ID NO: 11:

60

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 65 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both

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(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: other nucleic acid

5 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

10 (A) ORGANISM: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

15 CTATGATCCC TAATGGTGGG GCTTTTTTTAA GCCCACCATT AGGGAUCAUA GGCGCGTTTT 60
CGCGC 65

(2) INFORMATION FOR SEQ ID NO: 12:

20

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 67 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

25

(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

30

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

35 (A) ORGANISM: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

40 GTAATGCAGG AATAGCAATG CGTCCTTTTG GACGCAUUGC TATTCUGCA UUACGCGCGT 60
TTGCGCGC 67

(2) INFORMATION FOR SEQ ID NO: 13:

45

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 67 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

50

(D) TOPOLOGY: circular

55

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

5 GTAATGCAGG AATAGCAATG CGTTCTTTTG AACGCAUUGC TATTCCTGCA UUACGCGCGT 60
 TTCGCGC 67

(2) INFORMATION FOR SEQ ID NO: 14:

10 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 67 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: both
 (D) TOPOLOGY: circular

15 (ii) MOLECULE TYPE: other nucleic acid

 (iii) HYPOTHETICAL: NO

20 (iv) ANTI-SENSE: NO

 (vi) ORIGINAL SOURCE:
 (A) ORGANISM: oligonucleotide

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

 ACAGCAATGC GTTCACTTAC CGCTGTTTTTC AGCGGUAAGT GAACGCAUUG CUGUGCGCGT 60
 TTCGCGC 67

30

(2) INFORMATION FOR SEQ ID NO: 15:

 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 67 base pairs
35 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: both
 (D) TOPOLOGY: circular

40 (ii) MOLECULE TYPE: other nucleic acid

 (iii) HYPOTHETICAL: NO

 (iv) ANTI-SENSE: NO

45 (vi) ORIGINAL SOURCE:
 (A) ORGANISM: oligonucleotide

 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

50 ATAGCAATGC GTTCACTTAC CGCTGTTTTTC AGCGGUAAGT GAACGCAUUG CUAUGCGCGT 60
 TTCGCGC 67

55 (2) INFORMATION FOR SEQ ID NO: 16:

 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 89 base pairs
 (B) TYPE: nucleic acid

- 35 -

(C) STRANDEDNESS: both
(D) TOPOLOGY: circular

5 (ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

10 (vi) ORIGINAL SOURCE:

(A) ORGANISM: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

15 GTAATGCAGG AATAGCAATG CGTTCACCTCA CCGCTGTTTT CAGCGGUGAG TGAACGCAU 60

GCTATTCCUG CAUUACGCGC GTTTCGCGC 89

(2) INFORMATION FOR SEQ ID NO: 17:

20

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 67 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

25

(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: other nucleic acid

30 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

35

(A) ORGANISM: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

GGAATGCAGG AATAGCCATG CGTCCTTTTG GACGCAUCGC TATTCCUGCA UCCGCGCGGT 60

40 TTCGCGC 67

(2) INFORMATION FOR SEQ ID NO: 18:

45 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 67 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: circular

55 (iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: oligonucleotide

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

5 GGAATGCAGG AATAGCCATG CGTTCCTTTG AACGCAUCGC TATTCCTGCA UCCCGCGCGT 60
TTCGCGC 67

(2) INFORMATION FOR SEQ ID NO: 19:

10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 67 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
15 (D) TOPOLOGY: circular
(ii) MOLECULE TYPE: other nucleic acid
(iii) HYPOTHETICAL: NO
20 (iv) ANTI-SENSE: NO
(vi) ORIGINAL SOURCE:
(A) ORGANISM: oligonucleotide

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

ACAGCCATGC GTTCACTCAC CGCTGTTTTT AGCGGUGAGT GAACGCAUGG CUGUGCGCGT 60
30 TTCGCGC 67

(2) INFORMATION FOR SEQ ID NO: 20:

35 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 67 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: circular
40 (ii) MOLECULE TYPE: other nucleic acid
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
45 (vi) ORIGINAL SOURCE:
(A) ORGANISM: oligonucleotide

50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

ATAGCCATGC GTTCACTCAC CGCTGTTTTT AGCGGUGAGT GAACGCAUGG CUAUGCGCGT 60
TTCGCGC 67

55 (2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 89 base pairs

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: circular

5 (ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

10

(vi) ORIGINAL SOURCE:

(A) ORGANISM: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

15

GGAATGCAGG AATAGCCATG CGTTCACCTCA CCGCTGTTTT CAGCGGUGAG TGAACGCAUC 60

GCTATTCCUG CAUCCGCGC GTTTCGCGC 89

20 (2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 71 base pairs

(B) TYPE: nucleic acid

25

(C) STRANDEDNESS: both

(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: other nucleic acid

30

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

35

(A) ORGANISM: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

GGAATGCTCG AATCGCAATG CGGCCATTTT TAUGGCCGCA UUGCGATTCC AGCAUUCGGC 60

40

GCGTTTCGCG C 71

(2) INFORMATION FOR SEQ ID NO: 23:

45

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 71 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

(D) TOPOLOGY: circular

55

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM:

- 38 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

5 GGAATGCTGG AATCGCAATG CGGTCATTTT TAUGACCGCA UUGCGATTCC AGCAUCCGC 60
GCGTTTCGCG C 71

(2) INFORMATION FOR SEQ ID NO: 24:

10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 68 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: circular
15 (ii) MOLECULE TYPE: other nucleic acid
(iii) HYPOTHETICAL: NO
20 (iv) ANTI-SENSE: NO
(vi) ORIGINAL SOURCE:
(A) ORGANISM: oligonucleotide

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

CTGCAATGCG GTCATTGACA GCAGCTTTTG CUGCUGUCAA TGACCGCAUU GGCAGGCGCG 68
TTTCGCGC
30

(2) INFORMATION FOR SEQ ID NO: 25:

35 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 67 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: circular
40 (ii) MOLECULE TYPE: other nucleic acid
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
45 (vi) ORIGINAL SOURCE:
(A) ORGANISM: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

50 TCGCAATGCG GTCATTGACA GCAGCTTTTG CUGCTGUCAA TGACCGCAUU GCGAGCGCGT 60
TTCGCGC 67

55 (2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 91 base pairs

- 39 -

(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: circular

5 (ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

10

(vi) ORIGINAL SOURCE:
(A) ORGANISM: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

15

GGAATGCTGG AATCGCAATG CGGTCATTGA CAGCAGCTTT TGCUGCUGUC AATGACCGCA 60

UUGCGATTCC AGCAUCCGC GCGTTTCGCG C 91

20 (2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 67 base pairs
(B) TYPE: nucleic acid
25 (C) STRANDEDNESS: both
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: other nucleic acid

30 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
35 (A) ORGANISM: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

40 TCGCATTGAA CAGCTTCTT CAGGTTTTTA CCUGAAGAAA GCTGUUCAAU GCGAGCGCGT 60

TTCGCGC 67

45 (2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
50 (D) TOPOLOGY: circular

55 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

- 40 -

(A) ORGANISM: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

5

TTGTACCTTG GGAATGCAGG AACAGCCATG CGTCCACTC

39

(2) INFORMATION FOR SEQ ID NO: 29:

10

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 41 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: both

15

(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

20

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: oligonucleotide

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

TCCTCATGGC AGCTCCTTTA GCTCTTGGAG ACGTGGAGAT T

41

30

CLAIMS

1. A method of producing plants which exhibit an agronomically desirable trait comprising mutating or otherwise modifying *in situ* in a plant cell at least one gene which when modified is responsible for providing the said trait and regenerating from a cell exhibiting the said trait fertile morphologically normal whole plants, characterised in that a polynucleotide is introduced into the plant cell, the said polynucleotide comprising at least one region which is substantially complementary to at least one region in the gene, which gene region when mutated or otherwise modified provides for the agronomically desirable trait, the region in the said polynucleotide containing at least one base mismatch in comparison with the like region in the said gene, so that the region in the said gene is altered by the DNA repair/replication system of the cell to include the said mismatch.
2. A method according to the preceding claim, wherein - prior to the *in situ* mutation or modification, the plant cell is transformed with a gene providing for an agronomically desirable trait, and/or the cell is treated with a chemical mutagen.
3. A method according to either of claims 1 or 2, wherein at least one of the following regions of the gene is mutated or otherwise modified: promoter, RNA encoding sequence or transcription terminator.
4. A method according to any preceding claim, wherein the transcription activating region of the gene is mutated or otherwise modified *in situ*.
5. A method according to any preceding claim, wherein the said trait is herbicide resistance.

herbicides; dinitroaniline or other tubulin binding herbicides; herbicides which inhibit imidazole glycerol phosphate dehydratase; herbicides which inhibit

acetolactate synthase; herbicides which inhibit acetyl CoA carboxylase; herbicides which inhibit protoporphyrinogen oxidase; herbicides which inhibit phytoene desaturase; herbicides which inhibit hydroxyphenylpyruvate dioxygenase and herbicides which inhibit the biosynthesis of cellulose.

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7. A method according to any one of claims 2 to 6, wherein the plant cell is prior transformed with a gene providing for resistance to insects, fungi, and/or herbicides.
8. A method according to any preceding claim, wherein the protein encoding region of the gene encodes an enzyme selected from the group consisting of EPSPS, GOX, PAT, HPPD, ACC, ALS, BNX and protox.
9. A method according to the preceding claim, wherein the said at least one region of the polynucleotide consists of RNA.
10. A method according to the preceding claim, wherein the polynucleotide other than that comprised by the said at least one region consists of DNA.
11. A method according to any one of the preceding claims, wherein the polynucleotide consists of between about 30 and 250 nucleotides.
12. A method according to the preceding claim, wherein the polynucleotide consists of between 50 and 80 nucleotides.
13. A method according to any preceding claim, wherein the polynucleotide comprises between about 60 and about 150 bases and has an overall 'dumbbell' like shaped secondary structure looped around upon itself at either end and with a central 'rod' region of paired complementary DNA and RNA sequences.
14. A method according to any one of claims 8 to 13, in which the said gene encodes an EPSPS having at least the residues Thr, Pro, Gly and Ala at positions corresponding to 174, 178, 173 and 264 with respect to the EPSPS depicted in SEQ ID No. 2,

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wherein the said mismatch results in at least one of the following modifications in the EPSPS enzyme in comparison with the native sequence:

- (i) Thr 174 - Ile
- (ii) Pro 178 - Ser
- (iii) Gly 173 - Ala
- (iv) Ala 264 - Thr

wherein (i) Thr 174 occurs within a sequence comprising contiguously Ala -Gly-Thr-Ala-Met; (ii) Pro 178 occurs within a sequence comprising contiguously Met-Arg-Pro-Leu-Thr; (iii) Gly 173 occurs within a sequence comprising contiguously Asn-Ala-Gly-Thr-Ala; and (iv) Ala 264 occurs within a sequence comprising contiguously Pro-Leu-Ala-Leu-Gly.

15. A method according to any one of claims 8 to 14, wherein the mismatch results in replacement of the terminal Gly residue within the sequence motif Glu-Arg-Pro-AA1-AA2-AA3-Leu-Val-AA4-AA5-Leu-AA6-AA7-AA8-Gly- in a region of the EPSPS enzyme corresponding to that spanning positions 202 to 216 in SEQ ID No. 2 by either an Asp or Asn residue.

16. A method according to any preceding claim, wherein the plant cell is a cell of a plant selected from the group consisting of canola, sunflower, tobacco, sugar beet, cotton, maize, wheat, barley, rice, sorghum, tomato, mango, peach, apple, pear, strawberry, banana, melon, potato, carrot, lettuce, cabbage, onion, soya spp, sugar cane, pea, field beans, poplar, grape, citrus, alfalfa, rye, oats, turf and forage grasses, flax and oilseed rape, and nut producing plants insofar as they are not already specifically mentioned.

17. A method according to any preceding claim, wherein the plant cell is converted into a protoplast prior to the *in situ* mutation or modification of the gene, or transcriptional regulatory regions thereof, which when modified provides for the agronomically

18. Plants which result from the method of any preceding claim, the progeny and seeds of such plants, and plant material derived from such plants, progeny and seeds.

19. A method of controlling weeds in a field, the field comprising weeds and plants according to claim 18, the method comprising application to the field of a herbicide to which the said plants have been rendered resistant.

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20. A method according to the preceding claim, further comprising the steps of applying to the field insecticidally effective amounts of insecticides and/or fungicidally effective amounts of fungicides after the field has been treated with the herbicide.

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/GB 98/01499

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/54 C12N15/82 C12N15/90 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
X	WO 91 19796 A (BAYLOR COLLEGE MEDICINE) 26 December 1991 * see the whole document, esp. p.22 1.23-26, p.43-45, p.57 1.7-17 *	1-3,5,6, 16-19
X A	WO 91 04323 A (MONSANTO CO) 4 April 1991 * see esp. p.4-10 *	18,19 5-17,20
X A	WO 92 06201 A (MONSANTO CO) 16 April 1992 * see esp. p.4-11 *	18,19 5-17,20
X A	WO 97 04103 A (RHONE-POULENC AGROCHIMIE ;LEBRUN MICHEL (FR); SAILLAND ALAIN (FR);) 6 February 1997 * see esp. p.10 *	18,19 5-17,20



Further documents are listed in the continuation of box C



Patent family members are listed in annex

Special categories of cited documents

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P* document published prior to the international filing date but

not later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X* document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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